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Osteopontin as two-sided mediator of intestinal inflammation

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Abstract

Osteopontin (OPN) is characterized as a major amplifier of Th1-immune responses. However, its role in intestinal inflammation is currently unknown. We found considerably raised OPN levels in blood of wild-type (WT) mice with dextran sodium sulfate (DSS)-induced colitis. To identify the role of this mediator in intestinal inflammation, we analysed experimental colitis in OPN-deficient ($OPN^{-/-}$) mice. In the acute phase of colitis these mice showed more extensive colonic ulcerations and mucosal destruction than WT mice, which was abrogated by application of soluble OPN. Within the $OPN^{-/-}$ mice, infiltrating macrophages were not activated and showed impaired phagocytosis. Reduced mRNA expression of interleukin (IL)-1β and matrix metalloproteinases was found in acute colitis of $OPN^{-/-}$ mice. This was associated with decreased blood levels of IL-22, a Th17 cytokine that may mediate epithelial regeneration. However, $OPN^{-/-}$ mice showed increased serum levels of tumour necrosis factor (TNF)-α, which could be due to systemically present lipopolysaccharide translocated to the gut. In contrast to acute colitis, during chronic DSS-colitis, which is driven by a Th1 response of the lamina propria infiltrates, $OPN^{-/-}$ mice were protected from mucosal inflammation and demonstrated lower serum levels of IL-12 than WT mice. Furthermore, neutralization of OPN in WT mice abrogated colitis. Lastly, we demonstrate that in patients with active Crohn's disease OPN serum concentration correlated significantly with disease activity. Taken together, we postulate a dual function of OPN in intestinal inflammation: During acute inflammation OPN seems to activate innate immunity, reduces tissue damage and initiates mucosal repair whereas during chronic inflammation it promotes the Th1 response and strengthens inflammation.

Keywords: Th1 immune response • inflammatory bowel disease • inflammation • phagocytosis

Introduction

Osteopontin (OPN), also known as Eta-1 (early T lymphocyte activation-1), is a key cytokine promoting the release of interleukin (IL)-12 and hence inducing the development of a Th1 immune response [1, 2]. OPN has been cloned for the first time in 1986 and has long been considered a structural bone protein linking bone cells to the bone extracellular matrix. It belongs to a protein family called SIBLINGs (small integrin-binding ligand N-linked glycoprotein), whose genes share common expression in bone and tooth, and encode among others an RGD motif (amino acid sequence arg-gly-asp) [3]. It is both a membrane bound as well as a secreted protein, which binds to multiple receptors, depending on its phosphorylation state, such as the integrin receptor αVβ3, the CD44 variant isoforms CD44v6 and CD44v7 and components of the extracellular matrix such as collagen type I [4–6]. Intracellular OPN binding to CD44 variant isoforms establishes the survival signal by complex formation with the ERM (ezrin/radixin/moesin)

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Table 1 Patient group studied for OPN expression in serum, PBMC and/or lamina propria mononuclear cells (LPMC)

				Clinical treatment		
Diagnosis	***n***	**Age**	**Disease activity**	**Aminosalicylates**	**Aminosalicylates and steroids**	**Azathioprine**
Healthy	7		–	–	–	–
Non-IBD[$]	10		–	–	–	–
CD	33	18–70	15*	21	12	16
UC	23	18–69	19[#]	16	12	12

[$]Diverticulitis (6), lymphocyte colitis (2) and ischemic colitis (2); *CDAI > 150 and [#]CAI > 4.

proteins and by inducing the PI_3K/Akt signalling cascade [7, 8]. In its soluble form OPN is a strong chemoattractant and a pro-inflammatory cytokine that functionally activates dendritic cells and macrophages and induces their differentiation towards a Th1-polarizing phenotype [1]. OPN, produced by epithelial cells and mononuclear cells, enables IL-12 release through integrin engagement and dampens the IL-10 response through CD44 binding in macrophages [1]. It has been suggested that this binding of the same molecule to two different receptors on a cell differentially regulates the key cytokines towards type 1 immunity [1].

In inflammatory bowel disease (IBD), a dysregulated immune response to bacterial or food antigens plays a major role. Crohn's disease (CD) is characterized as a Th1 directed immune response with increased $CD4^+$ T-cell production of interferon (IFN)-γ and activated macrophages that secret tumour necrosis factor (TNF)-α and IL-12 [9]. In contrast, ulcerative colitis (UC) is associated with an atypical Th2 response mediated by a distinct subset of NK T cells that produce IL-13 and are cytotoxic for epithelial cells [10]. Recently, it has been shown that IL-12 induction by OPN could only be detected in lamina propria mononuclear cells (LPMC) from CD patients, but not in LPMC from the control group or UC patients, which further implements the role of OPN in a Th1 immune response [11].

Consequently, in $OPN^{-/-}$ mice activated mononuclear cells produce significantly more IL-10 and virtually no IL-12 [12]. In the mouse model deletion of OPN has been demonstrated to ameliorate the development of several autoimmune diseases including experimental autoimmune encephalomyelitis and collagen-induced arthritis, although contradictory reports have been published [2, 13–16].

To further clarify the pleiotropic function of OPN in immune responses our experiments were designed to assess the OPN function in the DSS colitis model in $OPN^{-/-}$ mice and in patients with CD.

Materials and methods

Patients

Healthy individuals, patients with non-IBD related colitis and patients with chronic IBD treated at the Charité University Medicine Berlin, were included in this study with approval from the institutional human research ethics committee. All patients with IBD had an established diagnosis of CD or UC based on standard clinical, endoscopic and histological criteria (Table 1). Disease duration, manifestation and therapy were evaluated in all patients. Disease activities were evaluated by the CD activity index (CDAI) and by the clinical activity index (CAI) for UC [17, 18]. CDAI $>$ 150 and CAI $>$ 4, were both considered to define active disease. Peripheral blood samples and endoscopic biopsies from areas of active inflammation or normal appearing colon mucosa were taken. Informed consent was obtained from all patients with approval from the institutional human research ethics committee following the Helsinki Guidelines.

Mice

OPN-deficient ($OPN^{-/-}$) mice, kindly provided by Drs. Susan Rittling and David T. Denhardt (Rutgers University, NJ, USA), were backcrossed with C57BL/6 mice for 10 generations. Intercrosses with C57BL/6 $CD44v7^{-/-}$ mice were performed to produce C57BL/6 $OPN^{-/-}$ $CD44v7^{-/-}$ double deficient mice [19]. Mice were bred and housed under specific pathogen-free conditions in the animal facility of the Forschungseinrichtung Experimentelle Medizin (Berlin, Germany). All experiments were performed with age- and sex-matched animals in accordance with institutional, state and federal guidelines.

Induction of acute and chronic DSS colitis in mice

Acute colitis in mice was established with 2.5% (w/v) DSS (MP Biomedicals, OH, USA) in tap water *ad libitum* for 7 days. Mice were killed 1 week after the last DSS feeding. In some experiments, recombinant OPN (10 μg in 200 μl phosphate buffered saline (PBS), R&D systems, Wiesbaden, Germany) was given i.p. daily from day 4 to day 7. In the chronic approach, the initial DSS application was followed by normal drinking water for 10 days and the treatment scheme was repeated four times. Control mice received tap water without DSS. Progression of colitis was evaluated by weight loss as well as loose and bloody stools. In some experiments an OPN antibody (antimouse-OPN-antibody, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was given i.p. (10 μg in 200 μl PBS) twice a week to wild-type (WT) mice. Mice were killed 2 weeks after the last DSS feeding. Post mortem the entire colon was excised and 1-cm segments each of the distal, transverse and proximal colon were fixed in 4% formaldehyde for histological analysis. Two- to 3-μm serial sections were stained with haematoxylin and eosin. Histological scoring was performed

in a blinded fashion by a pathologist as a combined score of inflammatory cell infiltration: (0) no inflammation; (1) increased number of inflammatory cells in lamina propria; (2) inflammatory cells extending into the submucosa and (3) transmural inflammatory infiltrates; as well as tissue damage: (0) no mucosal damage; (1) discrete epithelial lesions; (2) erosions or focal ulcerations and (3) severe mucosal damage with extensive ulcerations extending into the bowel wall. The combined histological score (inflammatory score) ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). The extent of inflammation was given as the percentage of inflamed mucosa of the colon sections. The colitis index comprises the product of the inflammatory score and the extent of inflammation [20].

Immunohistochemistry

Sections of 4 μm were cut, deparaffinized and subjected to a heat-induced epitope retrieval step before incubation with antibodies. Slides were incubated for 30 min. with a polyclonal rabbit antibody against inducible nitric oxide synthase (iNOS) (Calbiochem, Laufelfingen, Germany; 1:1000 dilution) or a macrophage specific antibody (F4/80, 1:50, eBioscience, San Diego, CA, USA) for 30 min. Alkaline phosphatase (K5005, Dako, Glostrup, Denmark) was developed using Fast Red as chromogen. For detection of iNOS, the EnVision peroxidase kit compatible only with rabbit primary antibodies (K 4010, Dako) was employed and for F4/80 a biotinylated donkey anti-rat secondary antibody (Dianova, Hamburg, Germany) was used followed by the streptavidin AP kit (Dako). Peroxidase was developed with a highly sensitive diaminobenzidine chromogenic substrate for approximately 10 min. Negative controls were performed by omitting the primary antibody.

ELISA analysis

Soluble human or murine mature OPN from serum samples and from cell culture supernatants was determined using OPN-specific ELISAs (Assay Designs, MI, USA). ELISAs for testing murine IFN-γ and IL-10 from supernatants of *in vitro* cultured mesenteric lymph node (MLN) cells (stimulated with anti-CD3/CD28 antibodies) were obtained from R&D Systems (Minneapolis, MN, USA). For the detection of antibodies against DSS mouse serum from WT and $OPN^{-/-}$ mice with acute and chronic colitis (diluted 1:10) was incubated on DSS (10 μg/ml) coated ELISA plates (Nunc, Wiesbaden, Germany) and developed using an HRP-conjugated goat-antimouse-antibody. Serum levels of IL-17 were detected with Elisa Max™ Set Standard (BioLegend Inc., San Diego, CA, USA) according to the manufacturers procedures. Serum levels of IL-22 (R&D Systems) and TNF-α (Biosource, Camarillo, CA, USA) were quantified by using ELISAs according to the manufacturers procedures.

Phagocytosis assay

Phagocytosis was investigated in human and murine whole blood samples. Where indicated, blood samples were incubated with 10 μg/ml RGD-peptide (Merck, Darmstadt, Germany), anti-CD44v7 antibody (10 μg/ml, clone VFF9, BenderMedSystems, Vienna, Austria), isotype control or recombinant OPN (100 and 500 ng/ml) (R&D Systems) for 1 hr. Heparinized blood or cell suspensions at 2×10^6 cells/ml were used. Fifty microlitres of blood or cell suspension were added to opsonized fluoresceine isothiocyanate (FITC)-labelled *E. coli* following manufacturers instruction (Phagotest kit, Orpegen Pharma, Heidelberg, Germany). After phagocytes had been challenged with bioparticles for 10–60 min., trypan blue was added to quench extracellular fluorescence before fluorescence activated cell sorting (FACS) analysis. Data were acquired on a FACSCalibur (BectonDickinson [BD], Heidelberg, Germany), and collected and analysed with CellQuest software (BD).

Real-time RT-PCR

Murine tissue samples, snap frozen in Invisorb lyzing solution (Invitek, Berlin, Germany) were homogenized during thawing by means of Ultraturrax tissue homogenizer (Jahnke and Kunkel, Staufen, Germany) and then treated with 4 mg/ml proteinase K for 1 hr (Clontech Laboratories, USA). Isolation of total cellular RNA from murine tissues was done by use of the Invisorb RNA kit II (Invitek, Berlin, Germany). mRNA was reverse transcribed and analysed in triplicate assays by TaqMan PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously [21, 22]. For detection of murine IL-22, IL-22BP, IFN-γ, IL-17A, IL-17F, IL-21, IL-12 p35, IL-23 p19, IL-1β, IL-6, MMP2, MMP9, MM10, S100A9 and DefCR5 appropriate assays including double-fluorescent probes in combination with assays for housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT) were developed by ourselves (IL-22, IL-22BP, IFN-γ) or purchased from Applied Biosystems. Expression levels were calculated relative to the data for HPRT obtained with the every matching assay.

RNA analysis

Total RNA of LPMC from $OPN^{-/-}$ and WT mice with acute and chronic inflammation *versus* healthy mice was isolated using the RNeasy kit from Qiagen (Hilden, Germany). Five hundred nanograms of total RNA were reverse transcribed as described elsewhere [23]. TLR2, 4 and 9 were detected using the primers as described [24]. NOD2 was amplified using the primers as described [25].

Statistical analysis

Statistic evaluation of the experimental data was done with a Students' t-test calculator software from GraphPad Inc. (San Diego, CA, USA) and the Pearson's correlation coefficient test (Systat Software, Inc., San Jose, CA, USA).

Results

OPN deletion aggravates the course of acute experimental colitis in mice

Assuming that OPN mediates inflammation, $OPN^{-/-}$ mice should be protected from experimental colitis. Surprisingly, in the acute colitis model, $OPN^{-/-}$ mice were even more susceptible to DSS colitis compared to WT mice and $CD44v7^{-/-}$ mice. Morphologically,

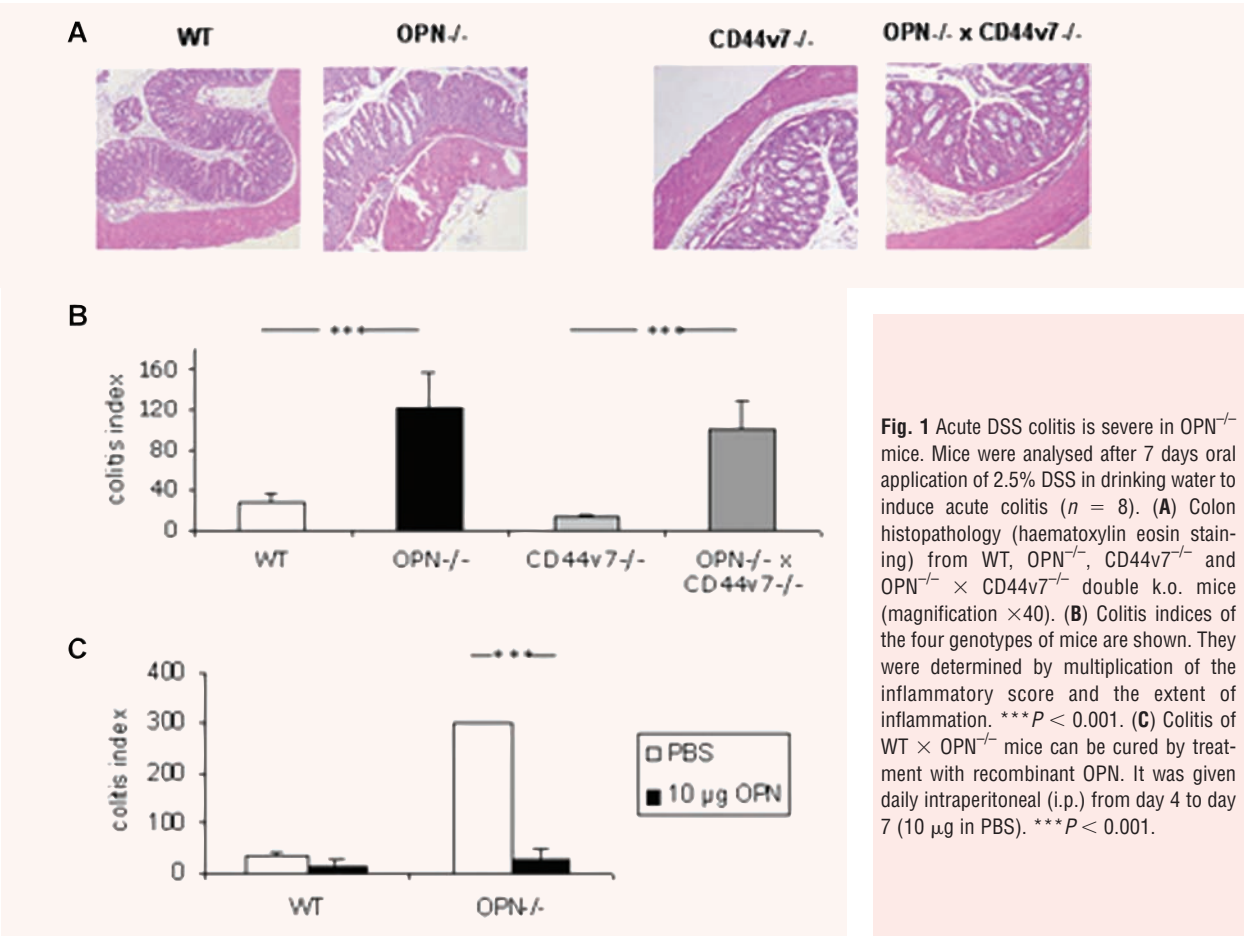

Fig. 1 Acute DSS colitis is severe in OPN$^{-/-}$ mice. Mice were analysed after 7 days oral application of 2.5% DSS in drinking water to induce acute colitis ($n = 8$). (**A**) Colon histopathology (haematoxylin eosin staining) from WT, OPN$^{-/-}$, CD44v7$^{-/-}$ and OPN$^{-/-}$ $\times$ CD44v7$^{-/-}$ double k.o. mice (magnification $\times$40). (**B**) Colitis indices of the four genotypes of mice are shown. They were determined by multiplication of the inflammatory score and the extent of inflammation. $^{***}P < 0.001$. (**C**) Colitis of WT $\times$ OPN$^{-/-}$ mice can be cured by treatment with recombinant OPN. It was given daily intraperitoneal (i.p.) from day 4 to day 7 (10 µg in PBS). $^{***}P < 0.001$.

OPN$^{-/-}$ and CD44v7 x OPN double-deleted mice showed extended inflammatory infiltrates and more severe ulcerations as compared to WT mice or CD44v7$^{-/-}$ mice, in which epithelial repair is detectable (Fig. 1A). Comparing the extensions of mucosal damage in WT and OPN$^{-/-}$ mice, inflammation was only focal in WT mice, whereas the whole colon was affected in OPN$^{-/-}$ mice. Colon length was reduced in OPN$^{-/-}$ (mean 4.3 $\pm$ 1.1) as well as in WT mice (5.4 $\pm$ 0.6) as compared to healthy control mice (9.5 $\pm$ 0.5) or CD44v7$^{-/-}$ mice (9.1 $\pm$ 0.2), but there was no significant weight loss in all groups. The histological examination revealed a significant increase in the inflammatory score of OPN$^{-/-}$ mice (5.5 $\pm$ 0.8) as compared to WT mice (3.2 $\pm$ 1.4). The colitis index (product of inflammatory score and extent of inflammation) in OPN$^{-/-}$ mice was four times higher (123 $\pm$ 35) than in the colon of WT mice (28 $\pm$ 8) (Fig. 1B). This was reflected by a higher extent of inflammation in the colon of these mice compared to WT animals: 8.0 $\pm$ 2.7% of the colon in WT mice was affected and 33 $\pm$ 9.7% in OPN$^{-/-}$ mice. Remarkably, although CD44v7 deleted mice are protected from experimental colitis (colitis index 14 $\pm$ 2), CD44v7 deletion was not protective in OPN$^{-/-}$ double deficient mice in acute DSS colitis (100 $\pm$ 28) (Fig. 1B). Hence, the effect of OPN deletion is more important than CD44v7 deletion in acute mucosal regeneration. Surprisingly, supplemental application of recombinant OPN ameliorated acute colitis in OPN$^{-/-}$ mice (28 $\pm$ 19, $P < 0.0001$) but had no significant impact on the colitis index in WT mice (Fig. 1C).

OPN deficient mice are protected from chronic experimental colitis

Analysing the chronic DSS colitis model, which more reflects the clinical situation of CD patients, we observed that OPN$^{-/-}$ mice showed no chronic infiltrates compared to the WT mice, which is consistent with a suppression of the Th1 immune response (Fig. 2A). The inflammatory activity was reflected by a reduced colon length in WT mice (7.3 cm $\pm$ 0.8), but not in OPN$^{-/-}$ mice (9.3 cm $\pm$ 0.5) and by an increased inflammatory score with

4.3 ± 0.3 in WT mice compared to 2.0 ± 0.2 in OPN$^{-/-}$ mice. The colitis index was 130 ± 12 (for WT) and 25 ± 5 (for OPN$^{-/-}$) ($P < 0.01$) (Fig. 2B). Furthermore, OPN$^{-/-}$ mice developed a significant anti-DSS-antibody response in chronic colitis compared to WT mice ($P < 0.05$), which indicates a stronger adaptive immune response (Fig. 2C). An anti-OPN antibody was applied to WT mice during the chronic approach, which significantly impaired inflammation, as demonstrated in the colitis index ($P < 0.007$) (Fig. 2D) and led to a decrease of the serum OPN levels (48 ng/ml, Fig. 2E). Additionally, we observed an enormous increase (5000-fold) of OPN serum levels in WT mice with acute (93 ng/ml) and chronic colitis (105 ng/ml) and in WT mice reconstituted with recombinant OPN (134 ng/ml) compared to the healthy control (20 pg/ml) (Fig. 2E). These results allude to a dichotomic function of OPN: Low concentration of OPN is required for tissue repair, but higher concentrations enhance Th1-driven immune responses.

OPN absence impairs activation of lamina propria macrophages during acute colitis

We compared the expression of iNOS within the inflamed mucosa in OPN$^{-/-}$ *versus* WT mice because OPN has been described to be involved in acute tissue repair [26]. iNOS, an indicator of inflammatory processes, is induced upon activation of antigen presenting cells [27]. Remarkably, whereas cell infiltrates were equally distributed in the inflamed lesions in WT and OPN$^{-/-}$ animals, we observed a clear induction of iNOS protein in the WT mice, but almost complete absence in OPN$^{-/-}$ mice (Fig. 3A). Furthermore, OPN$^{-/-}$ phagocytes have a significantly impaired capacity to perform phagocytosis (Fig. 3B) as detected in a phagocytosis assay with FITC-labelled opsonized *Escherichia coli*. Moreover, stimulation of phagocytes with recombinant OPN resulted in increased phagocytosis. Median doses of soluble OPN (100 ng/ml) but not high doses (500 ng/ml) rapidly (10 min.) increased phagocytic function (untreated cells: mean 10.1 ± 2.5; 100 ng OPN: mean 16.2 ± 2.9; 500 ng OPN: mean 9.9 ± 2.9), which was reduced by RGD peptide (mean 7.8 ± 3.0) and completely suppressed by a neutralizing anti-CD44v7 antibody (0.8 ± 0.2) (Fig. 3C), but not by an isotype control (anti-CD44v10: mean 12 ± 0.9). This effect was still significant after 30 min. revealing the importance of CD44v7 in phagocytic function. Then we analysed samples from the inflamed colons from 4- and 7-day DSS-treated WT and OPN$^{-/-}$ mice for mRNA expression of IL-1β, IL-6 and T-cell cytokines (IL-22, IFN-γ, IL-17A, IL-17F and IL-21) by real-time RT-PCR. In line with the observed diminished activation of lamina propria macrophages during acute colitis, the expression of IL-1β and IL-6 was less in OPN$^{-/-}$ mice than in WT mice (Fig. 3D). In these samples we found no differences in IL-22 expression between WT and OPN$^{-/-}$ mice (Fig. 3D), and, if at all we found very weak expression of IFN-γ, IL-17A, IL-17F and IL-21 (data not shown). It should be mentioned here that in OPN$^{-/-}$ macrophages, obtained from normal and inflamed gut mucosa, no significant differences in mRNA expression levels for TLR2, 4 and 9 as well as for NOD2 were detected (data not shown).

Lastly, we questioned the further consequences of the reduced activation of macrophages in inflamed colons of OPN$^{-/-}$ mice and analysed the expression of anti-bacterial proteins (Defcr5 and S100A9) and matrix metalloproteinases (MMPs) in the gut tissue. In doing so, we found diminished expression in particular of MMP2 but also of MMP10 and not MMP9 in the inflamed colons of 7-day DSS-treated OPN$^{-/-}$ mice (acute colitis) in comparison to the colons of WT mice (Fig. 3E).

OPN deficiency causes different systemic cytokines responses

In the next step we searched for possible systemic differences between WT and OPN$^{-/-}$ mice during acute and chronic DSS-colitis. First, we investigated the cytokine mRNA expression in MLNs. As demonstrated in Fig. 4A, we determined a reduced expression of Th17 cytokines (particularly of IL-22) during acute colitis in MLNs from OPN$^{-/-}$ compared to MLNs from WT mice. In contrast, there were no differences in IFN-γ mRNA expression between WT and OPN$^{-/-}$ in both 4- and 7-day DSS treated mice (Fig. 4A). Correspondingly, the OPN$^{-/-}$ mice showed reduced serum levels of IL-22 (Fig. 4B) at day 4 and IL-17A (Fig. 4C). Interestingly, the levels of TNF-α in these samples were elevated in OPN$^{-/-}$ in comparison to WT mice during acute DSS colitis (Fig. 4B). The application of recombinant OPN increased IL-17 blood levels in OPN$^{-/-}$ mice comparable to those of WT mice (Fig. 4C) (in these samples the levels of IL-22 and TNF-α were not evaluated).

Lastly, the secretion of IFN-γ and IL-10 was evaluated from MLN cells in WT and OPN$^{-/-}$ mice in acute (day 7) and in chronic colitis (day 26). In line with the mRNA results, no differences in the IFN-γ levels were observed in acute colitis in OPN$^{-/-}$ compared to WT mice. However, in the chronic DSS colitis, IFN-γ levels were significantly lower in OPN$^{-/-}$ mice ($P < 0.01$), whereas IL-10 secretion was increased ($P < 0.001$) compared to WT mice (Table 2). During the second, chronic, DSS treatment period at day 24, but not during the first DSS treatment period at day 0, we found a clear reduction of IL-12 mRNA expression in MLNs (Fig. 4D).

Serum OPN correlates with disease activity status in CD patients

OPN exists as an intracellular and a secreted protein. The latter circulates as soluble molecule or can be specifically bound to the cell surface *via* the OPN receptors. Levels of soluble OPN were determined in the serum of CD and UC patients with different actual disease activities (Fig. 5A). In patients with active CD the OPN serum levels were strongly higher (323 ± 81 ng/ml) than in the healthy control group (27 ± 11 ng/ml) and in patients with inactive CD (45 ± 9 ng/ml) ($P < 0.01$). Consequently, a direct

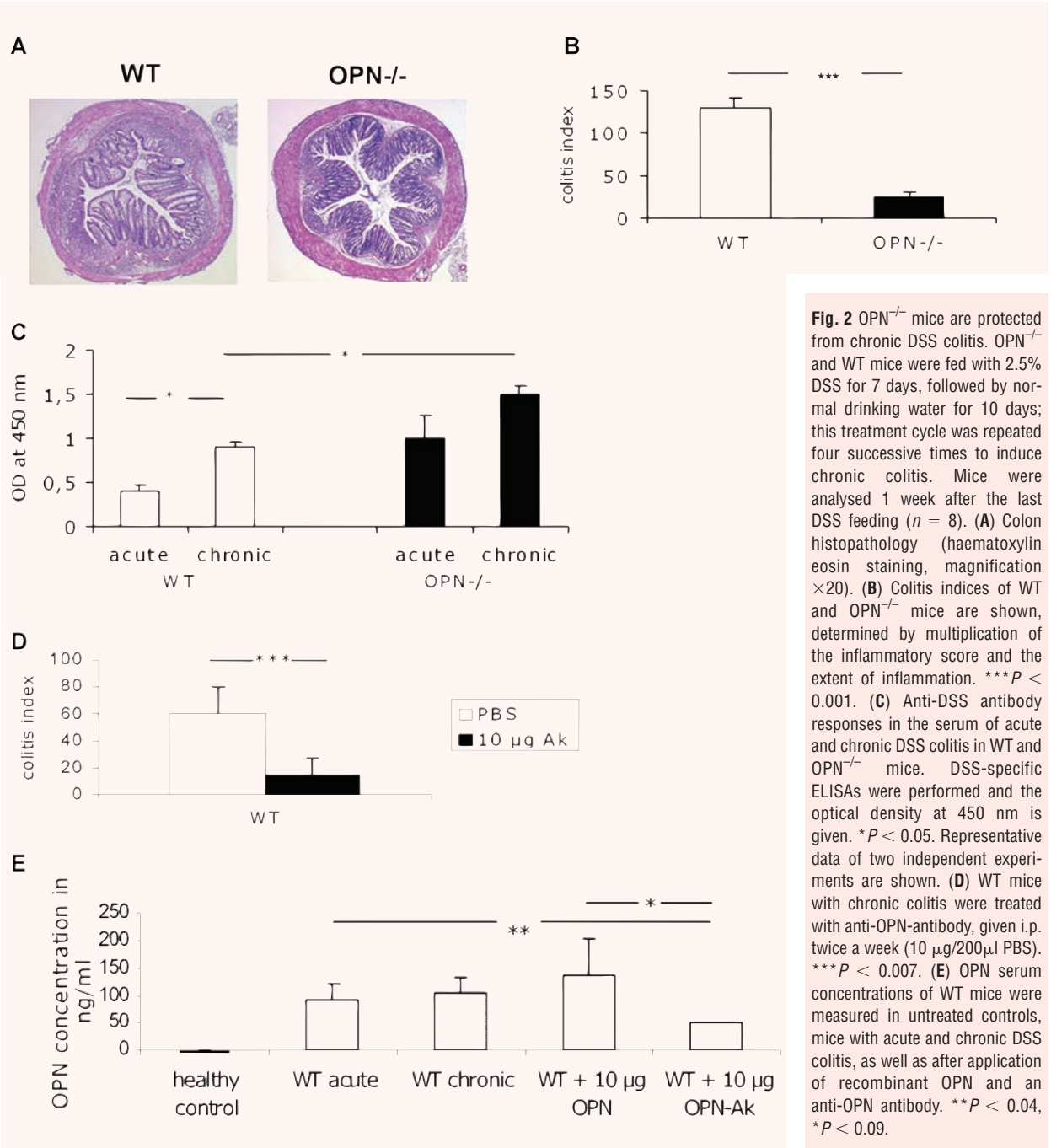

Fig. 2 OPN$^{-/-}$ mice are protected from chronic DSS colitis. OPN$^{-/-}$ and WT mice were fed with 2.5% DSS for 7 days, followed by normal drinking water for 10 days; this treatment cycle was repeated four successive times to induce chronic colitis. Mice were analysed 1 week after the last DSS feeding ($n = 8$). (**A**) Colon histopathology (haematoxylin eosin staining, magnification ×20). (**B**) Colitis indices of WT and OPN$^{-/-}$ mice are shown, determined by multiplication of the inflammatory score and the extent of inflammation. $^{***}P < 0.001$. (**C**) Anti-DSS antibody responses in the serum of acute and chronic DSS colitis in WT and OPN$^{-/-}$ mice. DSS-specific ELISAs were performed and the optical density at 450 nm is given. $^{*}P < 0.05$. Representative data of two independent experiments are shown. (**D**) WT mice with chronic colitis were treated with anti-OPN-antibody, given i.p. twice a week (10 µg/200µl PBS). $^{***}P < 0.007$. (**E**) OPN serum concentrations of WT mice were measured in untreated controls, mice with acute and chronic DSS colitis, as well as after application of recombinant OPN and an anti-OPN antibody. $^{**}P < 0.04$, $^{*}P < 0.09$.

correlation between CDAI and OPN concentration in CD ($r^2 = 0.29$, $P = 0.006$) was detected (Fig. 5B). OPN levels in UC patients were slightly elevated in patients with acute symptoms (140 ± 29 ng/ml) ($P < 0.05$) (Fig. 5A), compared to patients with inactive UC (126 ± 20 ng/ml), but no correlation between CAI and OPN serum level could be seen ($r^2 = 0.007$, $P = 0.73$) (Fig. 5B). In non-IBD colitis (76 ± 17 ng/ml) OPN levels were not increased. Medication with corticosteroids and immune modulators (azathioprine, methotrexate and infliximab) was not significantly different among patients with UC and CD. Further, disease duration and type of

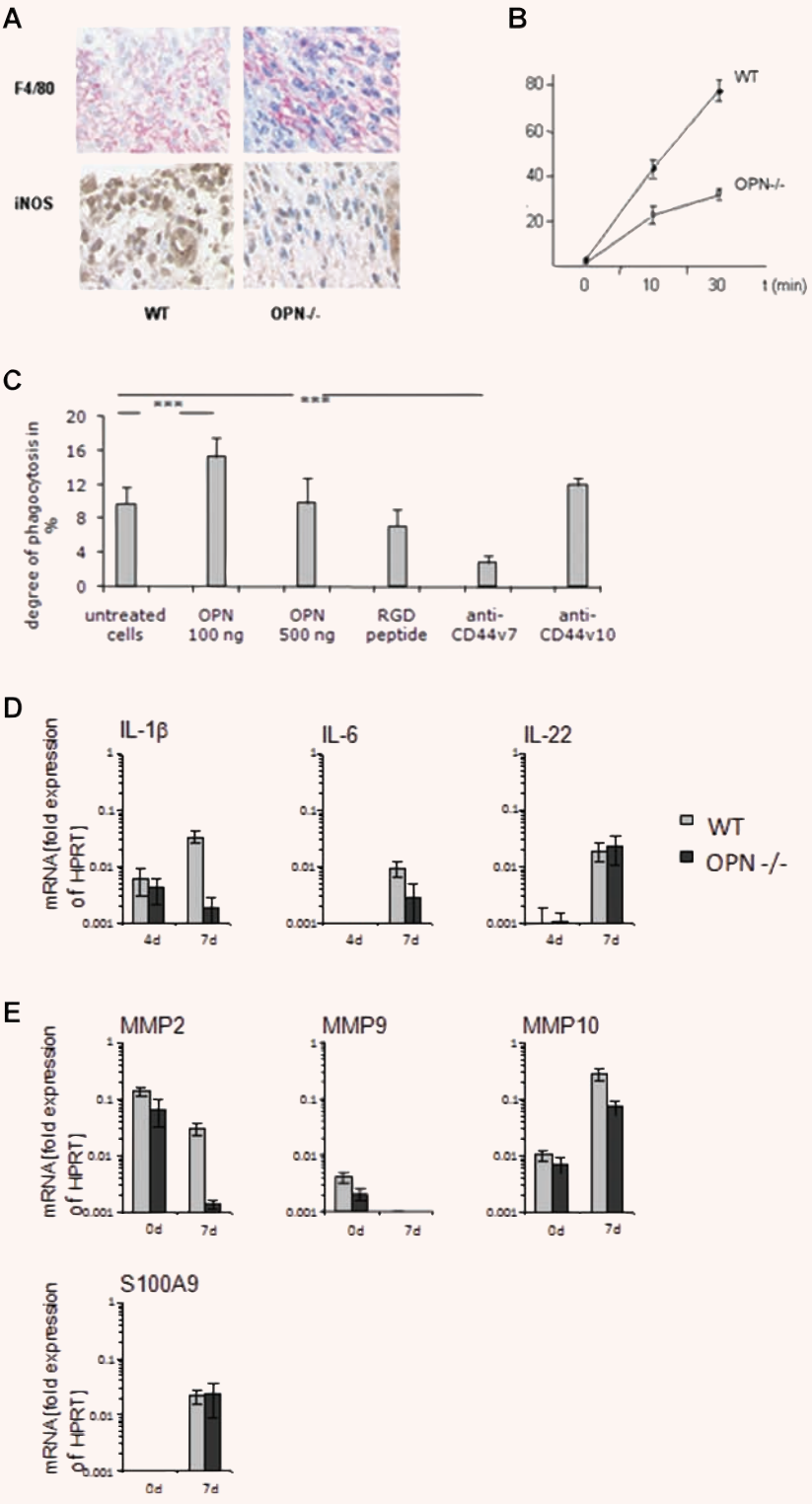

Fig. 3 OPN$^{-/-}$ mice with acute colitis show lack of macrophage activation, phagocytic capacity and reduced expression of IL-1β, IL-6 and MMPs. (**A**) Mice with acute colitis were analysed for iNOS expression by immunohistochemistry. Mature macrophages were equally present in the inflamed lamina propria of WT and OPN$^{-/-}$ mice, as shown by staining with F4/80 (specific for mature macrophages). Overt iNOS production by macrophages was observed in the mucosal infiltrates of WT animals, but was strongly reduced in OPN$^{-/-}$ mice (representative photomicrographs, original magnification ×600). (**B**) Macrophages, isolated from inflamed lamina propria of OPN$^{-/-}$ mice, showed reduced capacity to phagocyte opsonized FITC-labelled *E. coli* as compared to WT macrophages ($n = 2$). (**C**) In human macrophages, phagocytosis could be induced by low doses of soluble OPN (100 ng/ml), but not by high doses (500 ng/ml). $^{***}P < 0.0001$. This induction was down-regulated by RGD peptide (10 μg/ml) and completely abrogated by addition of anti-CD44v7 antibody (10 μg/ml) as compared to untreated cells or to an isotype control (anti-CD44v10; 10 μg/ml). $^{***}P < 0.001$. Representative data of two independent experiments are show. (**D**) Messenger RNA expression of IL-1β, IL-6 and IL-22 in the inflamed colon on day 4 and day 7 (acute colitis) was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M. (**E**) Messenger RNA expression of MMP2, MMP9, MMP10 and S100A9 in the inflamed colon on day 0 and day 7 was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M.

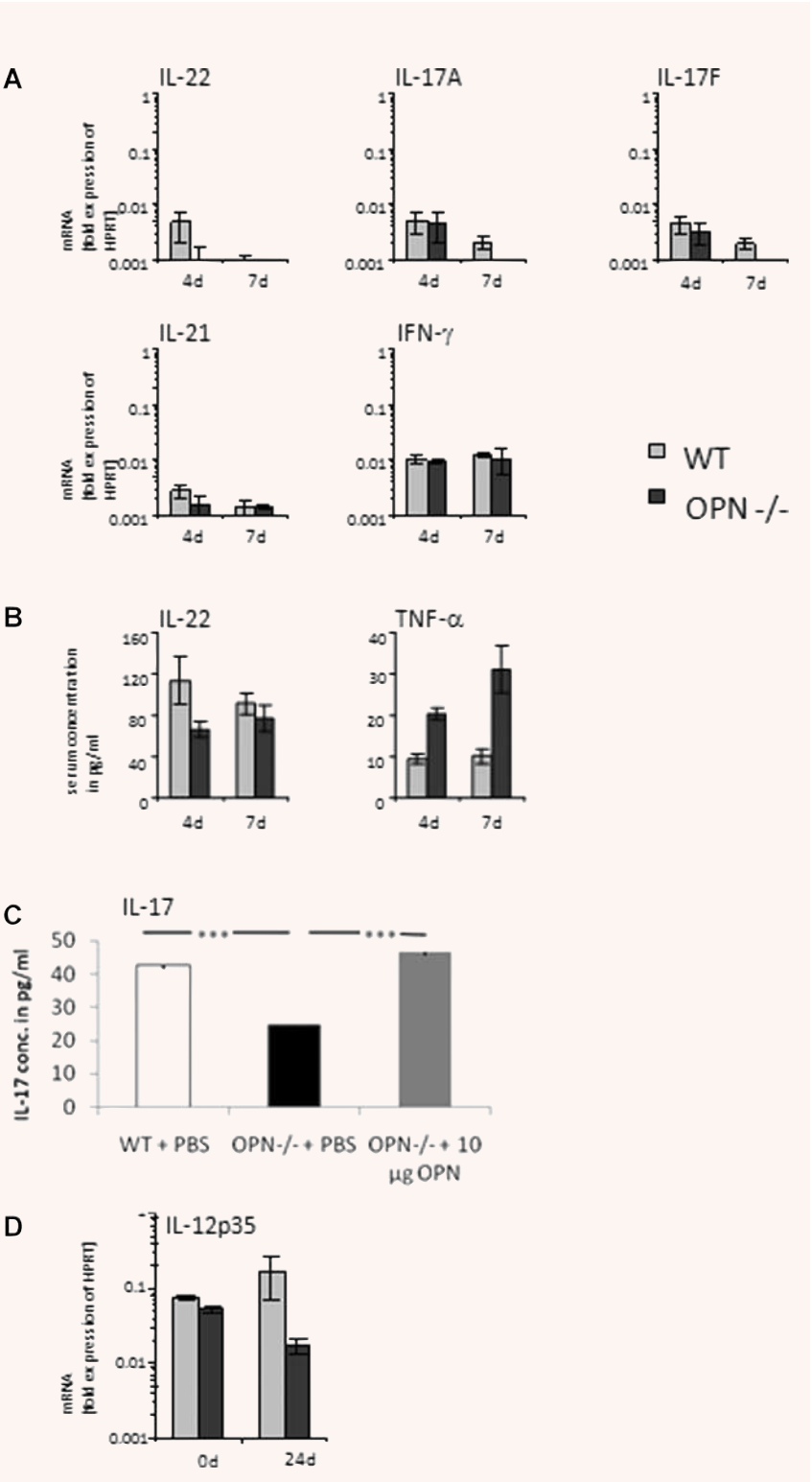

Fig. 4 OPN deficiency caused different systemic cytokines responses. OPN$^{-/-}$ and WT mice were fed with 2.5% DSS diluted in the drinking water for 7 days, followed by normal drinking water for 10 days and a subsequent second DSS treatment for another 7 days to induce chronic colitis. Mice were analysed before (day 0) or at day 4, day 7 or day 24 after start of DSS treatment. (**A**) Messenger RNA expression of IL-22, IL-17A, IL-17F, IL-21 and IFN-γ in mesenteric lymph node on day 4 and day 7 was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M. (**B**) Blood was collected on day 4 and day 7 for plasma recovery and analysed for the concentrations of IL-22 and TNF-α by ELISA. Data from five mice per group are given as the mean ± S.E.M. (**C**) Levels of IL-17 were determined in serum of WT and OPN$^{-/-}$ mice with acute DSS colitis. Recombinant OPN were given i.p. PBS served as control. $***P < 0.001$. (**D**) Messenger RNA expression of IL-12p35 in mesenteric lymph node on day 0 and day 24 was analysed by real-time RT-PCR as relative to HPRT expression. Data from three mice per group are given as the mean ± S.E.M.

Table 2 IFN-γ and IL-10 production in mesenteric lymph node cells from OPN$^{-/-}$ and WT mice

	OPN$^{-/-}$ mice		WT mice	
	IL-10	IFN-γ	IL-10	IFN-γ
	pg/ml		pg/ml	
Naïve mice	13.2 ± 0.5	562 ± 41	16.3 ± 3.5	499 ± 13
Acute colitis	18.5 ± 0.7	918 ± 56	14.2 ± 0.9	848 ± 103
Chronic colitis	75.0 ± 5.8*	601 ± 39	9.7 ± 1.1	1752 ± 124*

Results represent median ± S.E.M from four to six mice in each group.
*Significant differences in cytokine secretion over all groups ($P < 0.01$).

immunosuppressive therapy did not correlate with serum OPN in all patients. Thus, serum levels of OPN significantly correlate only with the CDAI of patients with active disease, irrespective of their treatment modalities.

Discussion

The complex function of OPN in IBD was examined in experimental colitis induced by DSS. This model comprises two characteristics of intestinal inflammation. First, oral administration of DSS causes acute colitis with severe mucosal ulcerations and infiltrations of innate immune cells. This acute colitis is accompanied by slow epithelial regeneration. Longer exposure to DSS then switches the colitis to chronic inflammation, characterized by large infiltrates of CD4$^+$ T cells in the lamina propria, whereas the epithelial layer regenerates after repeated rechallenge with DSS [28, 29]. Using this model, we identified two distinct effects of OPN in experimental colitis. In acute inflammation OPN$^{-/-}$ mice showed significantly more and extensive ulcerations compared to WT mice. Tissue damage could be cured by treatment with recombinant OPN in OPN$^{-/-}$ mice whereas OPN injections had no impact on the acute inflammation of WT mice. In chronic colitis, OPN$^{-/-}$ mice were protected from the T-cell-driven inflammation. In line with that, neutralization of OPN by an anti-OPN antibody completely abrogated chronic intestinal inflammation in WT mice. These data strongly support a dual function of OPN in experimental colitis.

We here demonstrate a cascade of inflammatory events: In acute colitis OPN deletion results in diminished activation of lamina propria macrophages with reduced expression of IL-1β; IL-6 and strongly down-regulated expression of MMP2 and MMP10 in the inflamed colon. We assume that the decrease of local repair mechanisms, in particular, of MMP2 lead to increased vulnerability of epithelial cells with decreased epithelial regeneration resulting in a defective barrier in the gut of OPN$^{-/-}$ mice compared to WT animals during acute colitis. In fact, in OPN$^{-/-}$ mice we showed that macrophages efficiently migrate into the inflammatory infiltrates, but the absence of iNOS staining demonstrated that these cells were not activated in the inflamed tissue. Furthermore, we found reduced phagocytosis of opsonized bacteria, and diminished oxidative burst in lamina propria macrophages from OPN$^{-/-}$ mice. In human macrophages median but not high doses of OPN led to a substantial increase in phagocytosis *in vitro*. Remarkably, phagocytosis was completely abrogated by antibody blockade of the OPN receptor CD44v7, a molecule highly involved in macrophage activation [30–32], but not by an RGD peptide that blocked the integrin receptor of OPN. The elimination of debris and pathogens by phagocytosis is essential in tissue repair and re-epithelialization. A defective phagocytosis thus should result in the persistence of neutrophilic infiltrates and tissue destruction [33, 34]. Our interesting observation that CD44v7 plays an important role in phagocytosis, and that neutralization or deletion of CD44v7 [31] alone protected mice from acute DSS colitis, but not double deletion of OPN plus its receptor CD44v7, suggests that OPN is essential for phagocytosis that is mediated *via* other receptors in the absence of CD44v7.

Coming back to the cytokine analysis we clearly show a reduced expression of Th17 cytokines, in particular IL-22, during acute colitis in MLNs and serum of OPN$^{-/-}$ compared to WT animals. IL-22 represents a novel type of immune mediator that regulates the function of tissue cells, *e.g.* in the skin, in the digestive and respiratory tract [35, 36]. Additionally, we recently described a protective systemic role of IL-22 in acute DSS colitis and probably in CD [37]. We and other groups have already demonstrated that IL-22 induces MMPs, protects tissue from damage and may lead to regeneration of epithelial cells [38–40]. It is also well documented, that IL-1°can induce some MMPs in *e.g.* fibroblasts [41]. In our study, the reduction of MMP2 was approximately five times more pronounced than that of MMP10 in OPN$^{-/-}$ mice in comparison to WT mice. Very interestingly, the group of Sitaraman demonstrated that selective deletion of MMP2 exacerbates both DSS and colitis induced by *Salmonella enterica subsp. Serovar Typhimurium* [42]. Additionally, the authors showed that mucosa-derived MMP2 was required for its protective effects

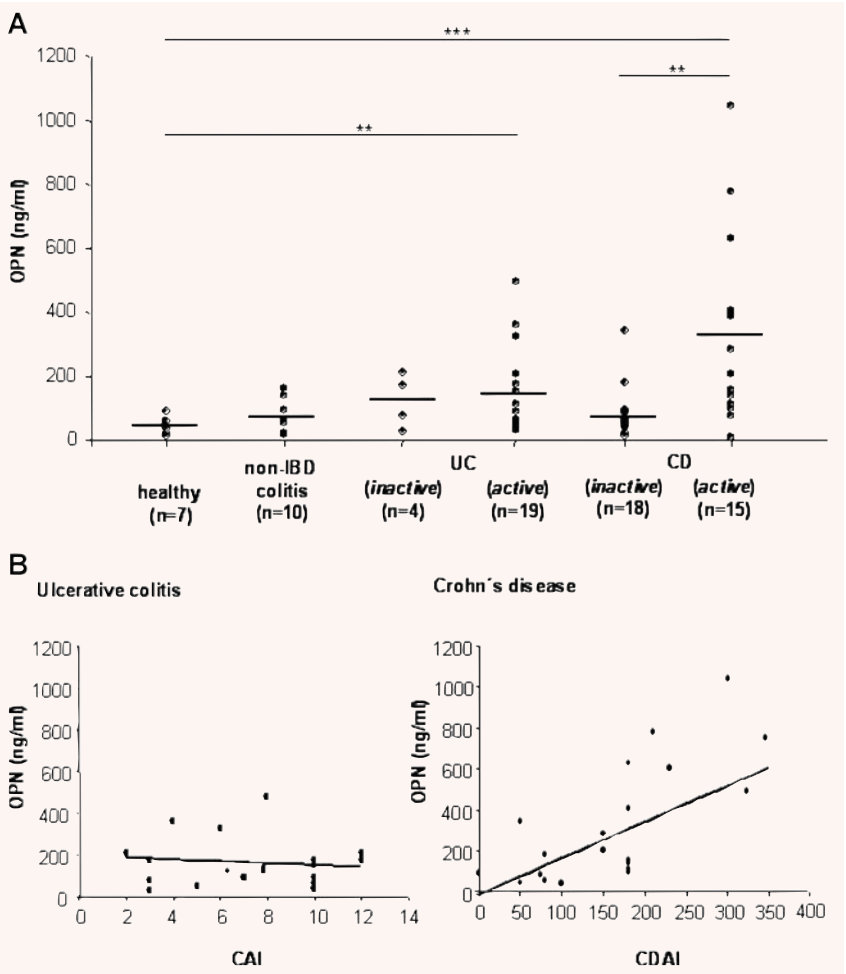

Fig. 5 Increased serum osteopontin correlates with disease activity in CD, but not in UC patients. (**A**) Serum from healthy volunteers and from patients with non-IBD colitis, inactive and active UC, inactive and active CD were collected. Concentration of OPN was evaluated by using an ELISA specific for human OPN. $^{**}P < 0.01$; $^{***}P < 0.001$. (**B**) Correlation between serum OPN concentration and disease activity was determined by Pearson's correlation coefficient test for patients with CD ($n = 33$) and UC ($n = 23$). A significant correlation between OPN concentration and CDAI was observed for patients with CD ($r = 0.54$; $r^2 = 0.29$; $P = 0.006$), but not for the CAI score in patients with UC ($r = 0.08$; $r^2 = 0.007$; $P = 0.73$).

towards colitis and that MMP2 regulates epithelial barrier function. It should be mentioned that deletion or therapeutic inhibition of MMP9 or MMP10 attenuates experimental colitis [43–45]. In the acute colitis, we detected elevated serum levels of TNF-α in OPN$^{-/-}$ mice, most probably induced by circulating lipopolysaccharide in these animals. In fact, in preliminary experiments we clearly detected lipopolysaccharide after 7 days of DSS treatment in the blood of four out of five OPN$^{-/-}$ animals and in none out of five WT animals. These results are suggestive of a defective barrier in the colons of OPN$^{-/-}$ mice in acute colitis and are supported by the decreased IL-22 serum levels in OPN$^{-/-}$ mice.

In WT mice, day 10 is the point of epithelial regeneration when DSS colitis shifts towards chronicity [28]. It is well demonstrated that Th1 cells play an important pathogenic role in chronic DSS colitis. OPN expression is regulated by T-bet, a transcription factor that controls cell polarization in Th1-mediated diseases [46]. Thus, opposed to acute inflammation, OPN$^{-/-}$ mice fully recovered between days 11 to 14. In chronic colitis we found clearly reduced IL-12 mRNA expression and IFN-γ secretion of LPMC was not increased in OPN$^{-/-}$ mice. Additionally, we demonstrate that the antibody response against DSS in WT mice with acute colitis was diminished significantly. In WT mice the shift towards a Th1 cytokine response might result in a decreased antibody secretion whereas OPN$^{-/-}$ mice demonstrate a Th2 directed immune response in colitis [11, 47]. The mouse data in our study very well match with the data in human CD, a Th1-mediated disease, where soluble OPN concentration in the serum strictly correlated with the CDAI in contrast to those of patients with colitis ulcerosa.

Our data now provide an interpretation of the conflicting data published on the impact of OPN in inflammation and autoimmune diseases [48–50]. The activities of OPN are not only confined to the adaptive immune responses, but OPN is part of an inherent protective mechanism, because recombinant OPN down-regulates acute inflammation in OPN$^{-/-}$ mice.

Very recently, additional dichotomic functions of OPN have been shown in allergic airway inflammation. In this study, administration of OPN at primary antigenic contact provided protection from allergic disease. This was mainly mediated through a shift

towards an anti-allergic Th1 response, as shown by increased levels of IL-12 and induction of regulatory dendritic cells whereas in the phase of antigen challenge OPN dampened the pro-allergic Th2 response [43]. Although most studies focused on the activity of secreted OPN as a Th1-directing cytokine, OPN is partially retained as intracellular OPN in the cytoplasm distinct from secretory vesicles also detected in the gut mucosa [51, 52]. Shinohara and coworkers have shown that especially intracellular OPN in dendritic cells is required for TLR9-dependent expression of IFN-α, which is supposed to be protective in inflamed mucosa [53]. Nevertheless, in our study OPN deletion has no significant effect on the expression of TLR2, 4 and 9.

However, the key question remains if targeting OPN is rather beneficial or deleterious in the treatment of IBD. Because the ongoing immune response is IL-12 driven and the epithelial damage is less significant in CD, neutralizing OPN might be a promising approach in this disease, but not in UC. In conclusion, the impact of OPN on intestinal homeostasis is reflected by two different functions. $OPN^{-/-}$ mice are protected from chronic experimental colitis by abrogating Th1 polarization, most probably as a consequence of abrogated receptor ligation on macrophages [1]. Furthermore, expression of OPN promotes macrophage activation *via* binding of CD44, induces a scavenger function and acts against acute tissue destruction. CD44v7 is the most plausible candidate as OPN receptor in colitis because CD44v7 is involved in Th1-driven intestinal inflammation, up-regulated on macrophages and necessary for macrophage maturation [54, 55].

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